

## Vaccinia Virus Entry, Exit, and Interaction with Differentiated Human Airway Epithelia<sup>▽</sup>

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**Variola virus, the causative agent of smallpox, enters and exits the host via the respiratory route. To better understand the pathogenesis of poxvirus infection and its interaction with respiratory epithelia, we used vaccinia virus and examined its interaction with primary cultures of well-differentiated human airway epithelia. We found that vaccinia virus preferentially infected the epithelia through the basolateral membrane and released viral progeny across the apical membrane. Despite infection and virus production, epithelia retained tight junctions, transepithelial electrical conductance, and a steep transepithelial concentration gradient of virus, indicating integrity of the epithelial barrier. In fact, during the first four days of infection, epithelial height and cell number increased. These morphological changes and maintenance of epithelial integrity required vaccinia virus growth factor, which was released basolaterally, where it activated epidermal growth factor 1 receptors. These data suggest a complex interaction between the virus and differentiated airway epithelia; the virus preferentially enters the cells basolaterally, exits apically, and maintains epithelial integrity by stimulating growth factor receptors.**

The threat of bioterrorism has raised concern that smallpox could reemerge as a significant disease entity (10, 21, 46). The large population of unvaccinated individuals, the many immunocompromised people, and the lack of good treatments suggest that variola virus, the causative agent of smallpox, has the potential for extensive and rapid spread. Thus, there is a renewed interest in understanding poxvirus pathogenesis.

Variola virus infection occurs following the inhalation of virus-laden airborne droplets (7, 12, 13a, 34). Virus replication within the lung and lymphoid system is followed by viremia (16, 41, 57). Patients remain asymptomatic during the 7- to 17-day-long incubation period, after which symptoms develop, including fever, malaise, and head and body aches, followed by oropharyngeal lesions and skin rash 2 to 4 days later. Sloughing of oropharyngeal lesions and respiratory tract release discharge infectious viral particles into the air, perpetuating human-to-human spread (14, 27, 52). The high mortality rate associated with smallpox results from the overwhelming toxemia leading to respiratory and/or cardiac failure. These observations indicate that infection involves viral entry and exit through the oropharyngeal-respiratory route and suggest that the airways may play a key role in the viral life cycle.

Because of the risks of experiments using variola virus, the limitations and difficulties associated with studies of nonhuman animals, and insufficient clinical information from humans, much of our knowledge about the interaction between poxvirus and the host has come from studies using vaccinia virus and cultured cells. Vaccinia virus is the orthopoxvirus used for

immunization against smallpox, and it is widely substituted for variola virus in the investigation of poxvirus biology (20, 28, 47). The vast majority of vaccinia virus studies have employed cultured cell lines. While much has been learned from this work, studies of cultured cell lines may not always accurately replicate the *in vivo* airway environment or the cellular response to infection. Use of cell lines in studies limits the ability to learn whether the virus enters and exits the epithelium from the apical or basolateral surface. In addition, while comparing cell culture models and human infection involves much uncertainty, there seem to be differences between predictions based on studies in cell lines and observations of the clinical course of smallpox. For example, vaccinia virus can cause relatively rapid cell lysis when it infects cultured cell lines, whereas cough and respiratory symptoms are not clinical characteristics of smallpox early during the course of disease (14, 27). This difference suggests that the cellular response to infection might differ *in vivo* and in cultured cell lines. Thus, an understanding of the pathogenesis of infection might benefit from a study utilizing human airway epithelia.

### MATERIALS AND METHODS

**Primary human airway epithelial cell culture models.** Human airway epithelia from tracheas and bronchi of lungs removed for organ donation were isolated and cultured at the air-liquid interface as described previously (22). Epithelia were studied at least 2 weeks after seeding, when they had differentiated and had a ciliated apical surface.

For explant studies, human bronchial epithelium was cut into small blocks (approximately 2 by 2 mm) and positioned on etched glass surfaces (Lab-Tek chamber slides; Nunc, Rochester, NY) coated with human placental collagen. The segments were immersed with a minimum volume (sufficient to keep the small tissue blocks wet but not floating) of 1:1 Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 5% fetal bovine serum. The cultures were left undisturbed for 3 days, after which culture medium was changed to 1:1 Dulbecco's modified Eagle's medium–Ham's F-12 medium supplemented with 2% Ultrosor G. Medium was then changed every 2 to 4 days.

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Cells proliferated from the explant segments over a period of 7 to 21 days, at which time they were used for infection studies.

**Viral production.** B5R-enhanced green fluorescent protein (GFP) (eGFP) (51) and VSC20 (5) viruses were a generous gift from Bernard Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Virus vp37-eGFP was constructed as follows: plasmid pRB21 (4) containing the coding sequence for vp37 (a generous gift from B. Moss) was linearized by digestion with PstI/NheI. Plasmid pEGFP from Clontech was digested with PstI/SpeI to release the eGFP coding sequence, which was gel purified and ligated into linearized pRB21, generating pRB21-eGFP (vp37 contains eGFP at the C terminus). Standard viral recombination was used to generate the vp37-eGFP virus. Briefly, HeLa cells were infected with RB12 (3), a vaccinia virus lacking the coding sequence for vp37 (a generous gift from B. Moss), for 2 h at 37°C. Thirty minutes prior to the end of infection, a calcium phosphate precipitate containing plasmid pRB21-eGFP was added. Virus and precipitate mixture were later aspirated, and cells were fed fresh medium and returned to 37°C for 48 h. To visualize and harvest plaques, cells were overlaid with soft agar and stained with neutral red. Visible plaques were collected and used to reinfect HeLa cells. Three rounds of infection were performed, generating pure populations of GFP-positive plaques, after which time vp37-eGFP virus was propagated, titers were determined, and virus was frozen at -80°C.

**Viral infections.** Explants of human airway epithelial cells were infected with vaccinia virus (B5R-eGFP) for 1 h at 37°C. After aspiration of virus, explants were refed medium and monitored for infection (GFP-positive cells) by fluorescence microscopy. Primary cultures of differentiated airway epithelia grown on permeable supports were infected with virus at different multiplicities of infection (MOIs) either apically or basolaterally. For apical infections, virus was applied directly onto the apical surface and incubated for 1 h at 37°C. Virus was then aspirated, the apical surface was washed several times with phosphate-buffered saline (PBS), and the cultures were fed fresh medium and returned to the incubator. For those cultures infected through the basolateral side, basolateral medium was removed, the Millicell insert was turned upside down, and virus was applied to the basolateral side for 1 h at 37°C. Virus was then aspirated, the inserts were turned rightside up, and the basolateral surface was washed several times before being fed fresh medium and returned to the incubator.

**Immunoprecipitations and Western blots.** Cell lysate was prepared by incubation in lysis buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM sodium chloride, 5 mM EDTA, 2 mM sodium vanadate [ $\text{Na}_3\text{VO}_4$ ], 10 mM sodium pyrophosphate, 100 mM sodium fluoride, phenylmethanesulfonyl fluoride, leupeptin, pepstatin, aprotinin) with 1% Triton X-100 at 4°C. The lysate was collected and homogenized, membranes were pelleted, and the soluble fraction was incubated with antibody for immunoprecipitation. Immunoprecipitated protein was complexed with protein G-Sepharose (Pierce, Rockford, IL), and the complex was pelleted and washed. Protein was eluted by incubation in 2× sample buffer (4% sodium dodecyl sulfate, 100 mM dithiothreitol, 20% glycerol, 0.005% bromophenol blue, 0.065 M Tris [pH 6.8]), boiled, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corporation, Billerica, MA). Polyvinylidene difluoride membrane was blocked in 5% bovine serum albumin, washed, and incubated with antibody. Following washes, incubation in horseradish peroxidase-conjugated secondary antibody (1:10,000; Amersham Pharmacia, Piscataway, NJ) allowed bound-antibody detection with SuperSignal solution (Pierce, Rockford, IL) and exposure to film (X-OMAT AR; Kodak Scientific Imaging Film, Rochester, NY).

**HeLa cell bioassay for erbB1 activation.** Airway surface liquid (ASL) was collected by washing the apical surface of multiple epithelia with PBS. Basolateral medium that had bathed the epithelia was also collected. These fluids were placed onto HeLa cells grown on 35-mm dishes to 80% confluence for 15 min at 37°C. As a positive control, HeLa cells were stimulated with recombinant human epidermal growth factor (EGF) (5 nM; R&D Systems, Minneapolis, MN) for 15 min. HeLa cells were lysed, and epidermal growth factor 1 (erbB1) protein was immunoprecipitated as described above.

**Measurement of transepithelial electrical conductance.** Transepithelial electrical conductance ( $G_t$ ) was measured with an ohmmeter (World Precision Instruments, Sarasota, FL). PBS was applied apically for each measurement and then removed.

**Immunocytochemistry and microscopy.** Epithelia were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (Pierce, Rockford, IL), washed, and blocked with Superblock blocking buffer (Pierce, Rockford, IL). Primary-antibody incubation of either 2 h at 37°C or overnight at 4°C was followed by washes, incubation with fluorophore-conjugated secondary antibody (1:200; Biomedex Corp., Foster City, CA), and counterstaining with ToPro3. Epithelia

were mounted onto glass slides, coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and studied using confocal microscopy (MRC-1024; Bio-Rad Laboratories, Hercules, CA). Epithelia used to examine morphology were fixed in zinc formalin overnight at 4°C, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard histological methods. Height was calculated using ImageJ analysis software and at least five images, with 15 measurements taken per image. The cell number was analyzed by counting nuclei in cross sections from at least five images from at least two different experiments.

**Antibodies.** Antibodies used for immunoprecipitation and Western blot analysis included mouse anti-erbB1 (BD Biosciences, San Jose, CA) and mouse anti-phosphotyrosine (Upstate Biotech, Billerica, MA). Antibody used for immunocytochemistry was a polyclonal rabbit or mouse monoclonal anti-ZO-1 antibody (1:100; Zymed, San Francisco, CA).

**Transmission electron microscopy.** To avoid disruption of ASL, samples were processed using an aqueous-free perfluorocarbon-based technique. Cell cultures were fixed for 2 h in 2% osmium tetroxide in FC-72 perfluorocarbon (3M, St. Paul, MN) and then dehydrated with three changes of 100% ethanol. The samples were then transitioned to Eponate 12 (Ted Pella, Redding, CA), embedded, and cured overnight at 65°C. Thin sections (70 nm) were picked up on 135-mesh hexagonal copper grids and poststained with Reynold's lead citrate and 5% uranyl acetate. Sections were imaged using a JEOL 1230 (Peabody, MA) transmission electron microscope equipped with a Gatan 2K-by-2K charge-coupled-device camera (Pleasanton, CA). Ten random fields of view were recorded from each condition. Images were analyzed with ImageJ (NIH) software, and three ASL height measurements were taken from each image.

## RESULTS

**Vaccinia virus preferentially infects differentiated airway epithelia through the basolateral membrane.** We first grew explants of human airway epithelia on collagen-coated glass substrates and infected them with a vaccinia virus that expresses GFP (51). Twenty-four hours later, we found nearly all of the GFP-positive cells located at or near the edges of confluent cell sheets, whereas we rarely observed GFP-positive cells within confluent cell sheets (Fig. 1). Selective infection of cells near the perimeter of cell islands suggested that vaccinia virus preferentially infects cells through the basolateral membrane, which is accessible near the edge of cell sheets.

To test this hypothesis directly, we studied well-differentiated primary cultures of human airway epithelia grown at the air-liquid interface (22). After seeding onto permeable filter supports, the cells differentiate into an epithelium containing ciliated, goblet, columnar, and basal cells.

We applied vaccinia virus to either the apical or basolateral surface for 1 h. Twenty-four hours following apical application, only a few GFP-positive cells were evident (Fig. 2A to C). Thus, vaccinia virus can infect from the apical surface but with low efficiency. In contrast, following basolateral application, infection was much more extensive (Fig. 2D to F).

As an assessment of epithelial junctions, we immunolocalized the tight junction protein ZO-1 (Fig. 2). Interestingly, vaccinia virus infection from either surface had little effect on the ZO-1 distribution, with the pattern of immunostaining appearing the same in infected and uninfected areas. This result suggested that infection may have caused little disruption of the cells or their tight junctions, at least for 24 h after infection. The lack of gross cellular destruction contrasts with the obvious toxicity caused by vaccinia virus infection of HeLa and Vero cultured cell lines. This difference suggested the presence of mechanisms that prevent epithelial barrier disruption following infection.

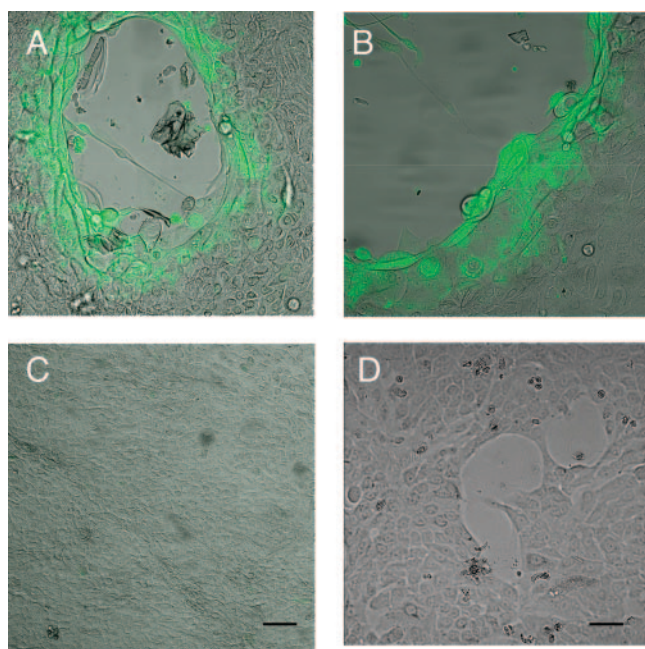


FIG. 1. Vaccinia virus infection of human airway epithelial explants. Explants grown on glass slides were infected with B5R-eGFP (MOI of 1 for 1 h) and analyzed 24 h later. (A and B) Areas of nonconfluent cells showing GFP-positive cells at edges of confluent areas. (C) Confluent areas of infected cells showed few, if any, GFP-positive cells. (D) Uninfected explant, which served as a control, lacked GFP expression. Scale bars indicate 100  $\mu$ m for A and C and 50  $\mu$ m for B and D.

**Vaccinia virus is preferentially released into the ASL.** To learn whether virus is preferentially released in a polar manner, we collected the basolateral medium and the ASL that covers the mucosal surface of infected epithelia and quantified virus with a plaque assay. By 72 h after infection, the ASL contained 25-fold more total PFU than the basolateral medium (Fig. 3A). These data indicate that vaccinia virus preferentially exits from the epithelia across the apical surface. Because the ASL volume is much smaller than the volume of basolateral medium, these data also suggested that the concentration of virus in ASL would be high. To calculate the volume of ASL, we used the area of the epithelium and transmission electron microscopy to measure ASL depth 3 days after infection. ASL depth was  $8.3 \pm 1.5$   $\mu$ m ( $n = 30$  from three epithelia), in good agreement with previous measurements (54). These measurements predict a virus concentration of approximately  $2.2 \times 10^7$  PFU/ml in ASL; this concentration is about 4 logs higher than that in the basolateral medium (Fig. 3B). The steep transepithelial virus concentration gradient suggests that epithelial integrity remained intact, at least for 72 h after infection.

**Virions produced by airway epithelia infect through the basolateral membrane.** Our data indicate that vaccinia virus produced by Vero cells infects predominately through the basolateral surface. Because of the complex life cycle of vaccinia virus and its production of different forms of infectious progeny (42, 44), we asked whether virions produced by airway epithelia and released apically would show a similar polarity of infection. Three days after infecting the epithelia, we collected

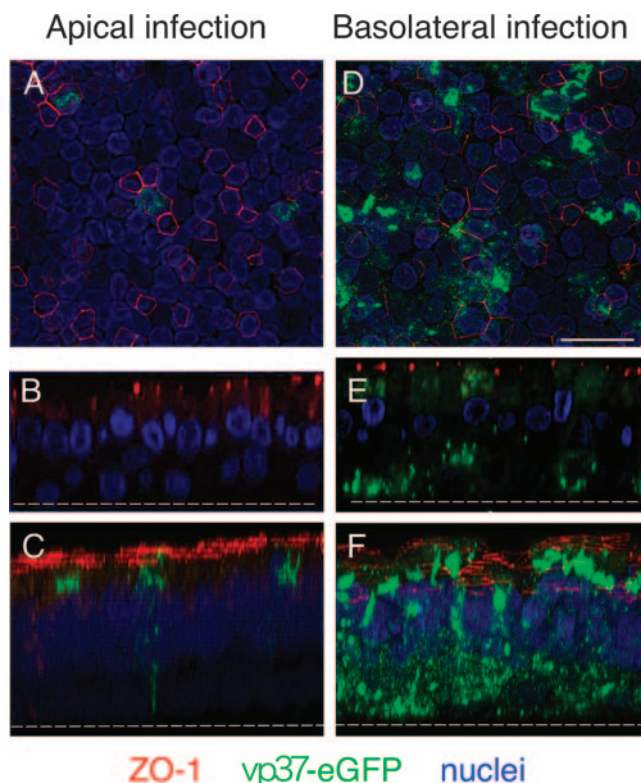


FIG. 2. Vaccinia virus infection of apical and basolateral surfaces of primary cultures of differentiated human airway epithelia. Epithelia were infected with the B5R-eGFP vaccinia virus (15 MOI for 1 h) from either the apical (A to C) or basolateral (D to F) surfaces. Twenty-four hours later, epithelia were processed and examined for eGFP fluorescence (green) and for immunolocalization of ZO-1 (red) and ToPro3 for nuclei (blue). (A and D) En face ( $x$ - $y$ ) stacked images of epithelium. (B and E) Single vertical ( $x$ - $z$ ) section. (C and F) Stacked vertical ( $x$ - $z$ ) sections from the epithelium. The scale bar indicates 20  $\mu$ m.

the ASL and applied it to the apical or basolateral surface of naïve airway epithelia. Just as with virus produced in Vero cells, the ASL infected primarily through the basolateral surface (Fig. 4A and B). Because virions produced by airway epithelia may resemble those involved in host-to-host transmission in humans, these data support the conclusion that vaccinia virus infects preferentially through the basolateral membrane.

**Vaccinia virus infection causes proliferation of airway epithelia.** The normal pattern of ZO-1 immunostaining and the epithelium's maintenance of a steep virus concentration gradient suggested that epithelia remained relatively intact during the first few days after infection. To further examine how vaccinia virus influenced differentiated airway epithelia, we examined their appearance over time. Hematoxylin- and eosin-stained sections revealed a progressive increase in epithelial height so that by 96 h after infection, the height had nearly doubled (Fig. 5A and B). During this time, infection also increased cell number by one-third (Fig. 5C). We observed a similar response with both apical and basolateral infection. Thus, in contrast to the destruction that vaccinia virus causes in some cultured cell lines, it stimulated proliferation and maintained the differentiation of human airway epithelia.



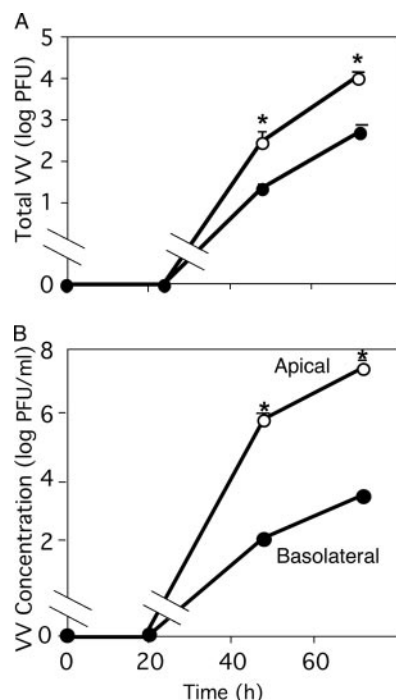
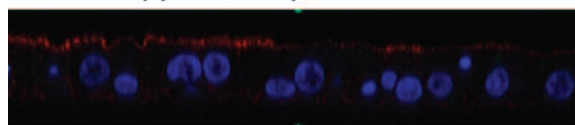


FIG. 3. Release of vaccinia virus (VV) from differentiated airway epithelia. Epithelia were infected with vp37-eGFP (MOI of 20 for 1 h) and basolateral medium, and ASL was collected at the indicated times. Collected ASL and medium were used in a plaque assay on Vero cells to determine amount of virus. (A) Total virus released from each surface. (B) Concentration of virus estimated based on the volume of basolateral medium and estimated ASL volume based on the area of epithelium and measured height of ASL ( $n = 5$ ). Asterisks indicate a  $P$  value of  $<0.0002$ .

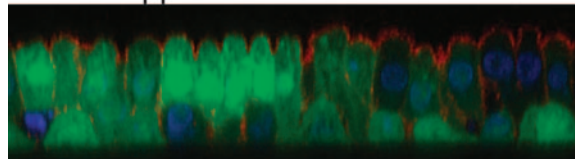
**VGF stimulates epithelial proliferation.** Vaccinia virus contains two copies of the vaccinia growth factor (VGF) gene, one at each end of its genome, flanked by inverted terminal repeats (5). Previous studies showed that VGF, an epithelial growth factor-like peptide, binds to and activates erbB1 (also called the EGF receptor) (48), induces cellular proliferation of infected cells (5, 6), and was required for viral replication (5, 26). Variola virus produces the closely related smallpox growth factor that also binds erbB1 (24). Thus, we hypothesized that the increase in cell number following infection was due to VGF. We infected well-differentiated epithelia with virus that retains the coding regions for VGF (vp37-eGFP) or a mutant virus with the two *VGF* genes disrupted (VSC20) (5). By 96 h after infection with the VSC20 virus, epithelia appeared irregular, and proliferative changes were not apparent (Fig. 6A). Thus, in contrast to effects with the vp37-eGFP virus, without VGF, the epithelia failed to hypertrophy even though infection with the two viruses was approximately the same (Fig. 6B and data not shown).

These morphological changes suggested that VSC20 infection would compromise epithelial integrity. We tested this hypothesis by measuring transepithelial  $G_r$  (Fig. 6C). Even 96 h after infection, vp37-eGFP had not altered the  $G_r$ . However, at 3 and 4 days after infection, the VGF mutant virus increased  $G_r$ . These data suggest that VGF production induces epithelial

#### A ASL applied to apical surface



#### B ASL applied to basolateral surface



Actin VP37-eGFP nuclei

FIG. 4. Vaccinia virus produced by airway epithelia preferentially infects epithelia from the basolateral surface. Differentiated epithelia were infected basolaterally with vaccinia virus (vp37-eGFP). Three days later, ASL was collected and used to infect naïve epithelia from the apical (A) or basolateral (B) surface. Three days later, cultures were stained with phalloidin-Alexa (red) to stain actin and ToPro3 to stain nuclei (blue) and analyzed for GFP expression (green).

proliferation and helps maintain epithelial integrity in the face of vaccinia virus infection.

**VGF activates the basolateral erbB1 receptor to stimulate proliferation.** Earlier studies showed that VGF can induce erbB1 phosphorylation and thereby activation (25). We found that erbB1 was phosphorylated in vp37-infected epithelia, whereas virus lacking VGF production (VSC20) failed to phosphorylate the receptor (Fig. 7). These results suggest that the activation of this growth factor receptor was a consequence of VGF secretion. Because previous work showed that erbB1 resides on the basolateral membrane of airway epithelial cells (39, 49), the data suggested that infected epithelia release VGF into the basolateral medium.

To further assess the polarity of VGF secretion, we collected ASL and basolateral medium from infected epithelia and applied them to HeLa cells as a bioassay for the presence of VGF. Figure 8 shows that basolateral medium from infected but not control epithelia stimulated erbB1 phosphorylation. In contrast, ASL caused no stimulation. These results suggest that infected epithelia preferentially release VGF from the basolateral surface. Thus, the ligand is secreted where it can readily access its receptor on epithelial cells.

These results implicate VGF as being an important link between vaccinia virus infection and the epithelial response. To further test this connection, we infected epithelia with vp37-eGFP virus and blocked erbB1 activation with the receptor tyrosine kinase inhibitor GW2974 (37). Compared to vehicle-treated controls, the GW2974-treated epithelia were not as tall (Fig. 9A). As an additional test, we applied exogenous EGF to epithelia infected with the VSC20 virus and found that it rescued the morphological changes resulting from the loss of VGF (Fig. 9B). These morphological changes are quantified in Fig. 9C. The effects were not due to different infection levels, because confocal images revealed similar numbers of infected cells (not shown). These results suggest that VGF stimulation of the epithelial erbB receptor is required and sufficient for the

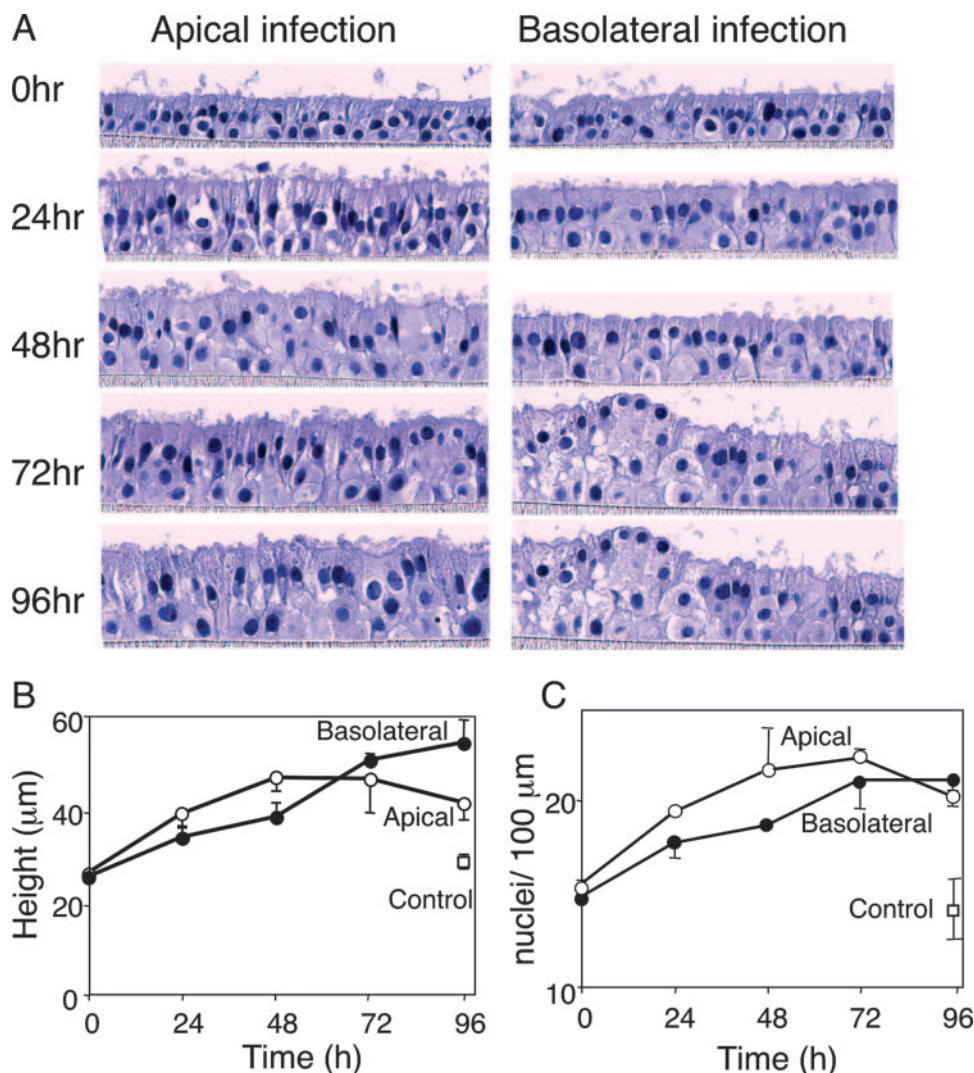


FIG. 5. Effect of vaccinia virus infection on airway epithelia. Vaccinia virus (B5R-eGFP) (MOI 0.5) was applied to the apical or basolateral surface for 1 h, and epithelia were processed at the indicated times. (A) Hematoxylin- and eosin-stained sections. (B) Epithelial height. (C) Number of nuclei/100  $\mu\text{m}$ .

vaccinia virus-induced morphological changes in human airway epithelia.

## DISCUSSION

Our results reveal specific interactions of vaccinia virus with differentiated human airway epithelia. Virus infection was more efficient through the basolateral membrane, whereas virus release occurred predominantly through the apical membrane. In addition, the virus produced VGF, which activated basolateral erbB1 receptors to stimulate epithelial hypertrophy and maintain epithelial integrity.

**Vaccinia virus infection through the basolateral membrane.** The ability of vaccinia virus to infect most cell lines has suggested a broad cellular tropism and possibly a ubiquitously expressed viral receptor (18, 29). However, the finding that vaccinia virus readily infected activated but not resting T cells suggested the presence of a specific cell surface viral receptor

(9). In addition, recent studies found that vaccinia virus interacts with glycosaminoglycans that may be cell type specific and attached to several different receptors (8, 19, 31). Our data showing greater infection from the basolateral surface than at the apical surface are consistent with a previous study of MDCK epithelia (35) and support the conclusion that vaccinia virus uses a specific receptor or receptors that localize preferentially on the basolateral membrane. Although the identity of the vaccinia virus receptor(s) remains uncertain, basolateral localization suggests a criterion and perhaps a strategy for its identification.

It is interesting that the natural route of infection of vaccinia virus is through the airway, and yet basolateral infection was more efficient than apical infection. Several other respiratory viruses, including adenovirus and measles virus, also initiate infection through a basolateral route (40, 56). For example, the receptor for adenovirus, the coxsackievirus-adenovirus receptor, localizes to the basolateral membrane, and as with vaccinia

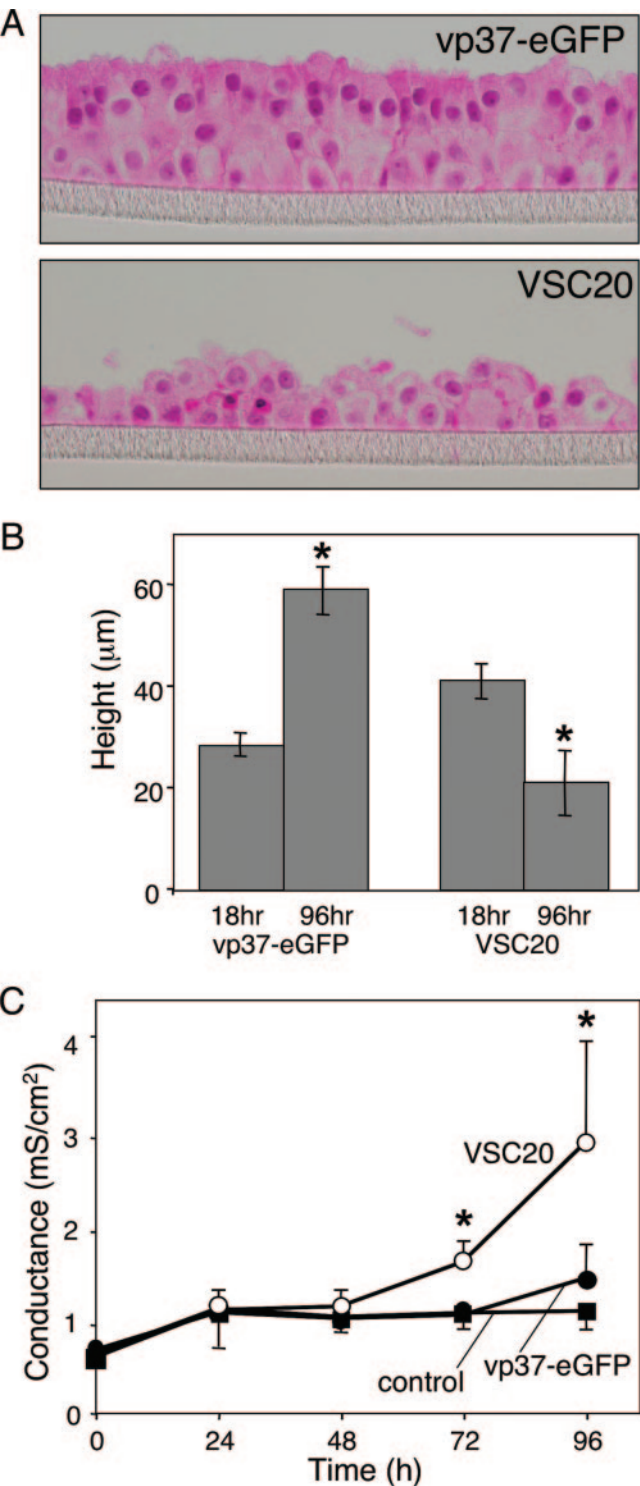


FIG. 6. Influence of vaccinia virus lacking VGF on airway epithelia. Epithelia were infected with vp37-eGFP or the VGF mutant VSC20 (MOI of 20 for 1 h). (A) Hematoxylin and eosin staining 96 h after infection. (B) Epithelial height measured 18 and 96 h after infection. (C). Transepithelial electrical conductance measured at indicated times. Asterisks indicate  $P$  values of  $<0.01$ .

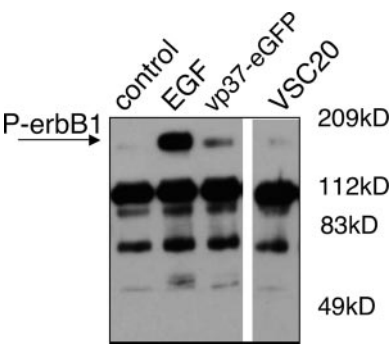


FIG. 7. erbB1 phosphorylation in vaccinia virus-infected epithelia. Differentiated airway epithelia were infected with vp37-eGFP or VSC20 (VGF-negative) virus (MOI of 20 for 1 h). Controls included uninfected cultures (control) and uninfected epithelia with recombinant EGF (5 nM) added basolaterally. Seventy-two hours after infection, epithelia were lysed, and erbB1 was immunoprecipitated and probed with an anti-phosphotyrosine antibody.

virus, adenovirus infection of differentiated airway epithelia is more efficient through the basolateral than the apical membrane (50). We speculate that naturally occurring vaccinia virus infection of humans might occur either at a low level through the apical membrane or through small breaks in the epithelium that provide access to the basolateral membrane. Alternatively, it is possible that infection occurs first through the lymphatic system rather than airway epithelia. Previous work provided evidence for both routes (16, 17, 33, 41, 57). We also wonder if the increased efficiency of basolateral compared to apical infection might facilitate infection of airway epithelia during the secondary viremia that occurs after replication in the lymphoid system and prior to extracellular shedding.

**Release of vaccinia virus into the ASL.** After gaining entry into a host cell, vaccinia virus begins transcribing early mRNAs (23). These transcripts generate cytoplasmic viral factories surrounded by endoplasmic reticulum membrane. In cultured cell lines, progeny virions move towards the cell surface along

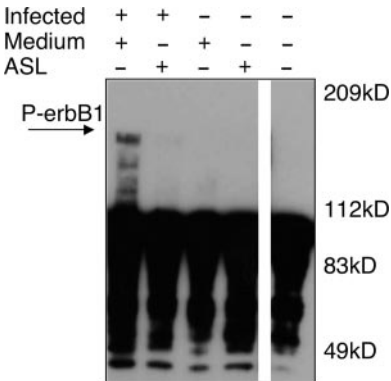


FIG. 8. Assay of ASL and basolateral medium for VGF. Differentiated epithelia were infected with vp37-eGFP (MOI of 20 for 1 h), and basolateral medium and ASL were collected 72 h later. Medium and ASL from uninfected epithelia were also collected. These liquids were applied to HeLa cells, and erbB1 was immunoprecipitated and probed with an anti-phosphotyrosine antibody. The apparent lack of erbB ligands in ASL from controls reflects the dilution required for the bioassay.



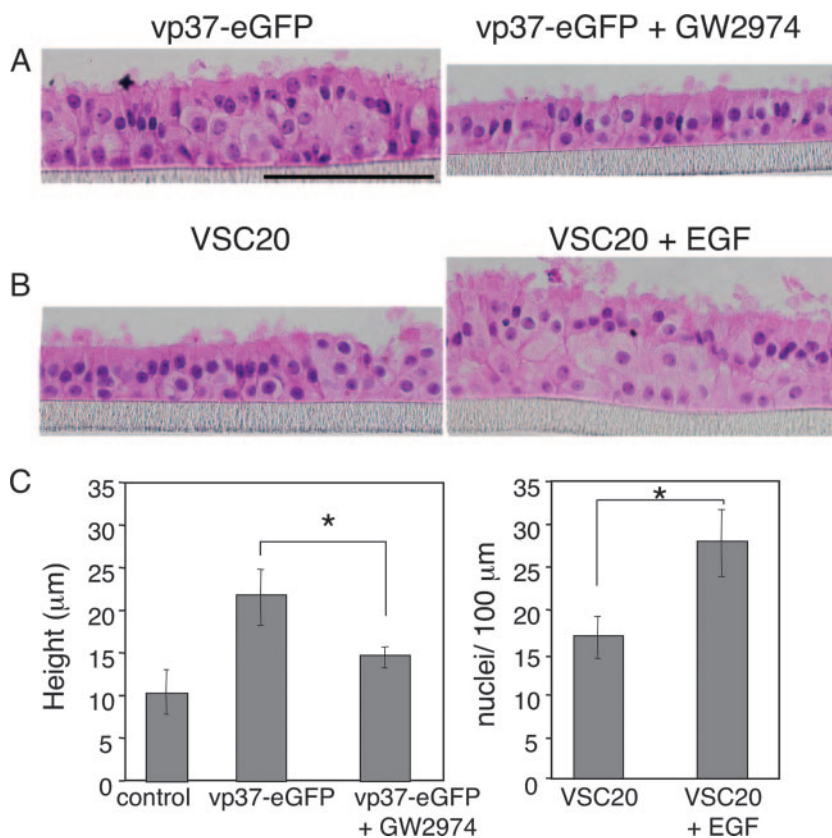


FIG. 9. Influence of VGF on response of airway epithelia to vaccinia virus. (A) Epithelia were infected with vp37-eGFP (MOI of 20 for 1 h) and treated with or without an erbB1/2 inhibitor (GW2974) (5  $\mu$ M) for 96 h. Epithelia were examined by hematoxylin and eosin staining. (B) Epithelia were infected with the VGF mutant virus VSC20 (MOI of 20 for 1 h) and treated with or without recombinant EGF for 72 h. (C) Epithelial height and number of nuclei/100  $\mu$ m for experiments shown in A and B. For height, 30 measurements from six images each from two different epithelia were made ( $n = 9$  for number of nuclei). Asterisks indicate a  $P$  value of <0.005. The scale bar indicates 50  $\mu$ m.

microtubules and are propelled out of the cell along actin tails (2, 11, 43). Interestingly, an actin web lies beneath the apical membrane (13, 15), the site of virus release in airway epithelia. We found much lower levels of virus in the basolateral medium. Although basolateral virus may have represented virus exit across the basolateral membrane, we cannot exclude the possibility that virus was released apically and then moved to the basolateral surface, driven by its steep concentration gradient. In addition, basal cells, which do not contact the apical surface, may have been a source of virus in the basolateral medium.

Consistent with our findings, some other viruses are also released across the apical membrane: coronavirus, influenza virus, and measles virus also exit apically from airway epithelia (30, 32, 36, 38, 40). Apical release may facilitate viral spread to other hosts without disrupting epithelial integrity or the tight junctions. A cough or sneeze could aerosolize droplets of ASL containing infectious virus and spread them to the environment.

**Role of VGF in maintaining epithelial integrity and hypertrophy.** Following vaccinia virus infection, cultured Vero and HeLa cells rapidly die. In contrast, virus-infected human airway epithelial cells retained several features of the differentiated epithelium. Transepithelial electrical conductance did not increase, the epithelium was able to maintain a transepithelial

viral concentration gradient, and the tight junction protein ZO-1 showed a normal expression pattern. In addition, airway epithelia were spared from extensive lysis, and instead, cell number and epithelial height increased following infection. Our data indicate that infected cells secrete VGF, which preserved epithelial integrity. This conclusion was supported by the findings that a VGF mutant virus failed to maintain transepithelial conductance, the VGF mutant failed to induce epithelial proliferation, exogenous EGF rescued the phenotype of the VGF mutant, and an erbB1 inhibitor mimicked the morphological changes observed with the VGF mutant virus. We wonder if maintenance of epithelial integrity despite infection may have a parallel in the clinical observation that patients do not develop pulmonary symptoms until late in the course of disease.

It is interesting that infected epithelia secrete VGF basolaterally. Previous studies showed that airway epithelia secrete EGF and several other growth factor ligands in the opposite direction, that is, into the ASL, where they are segregated from their basolateral receptors (49). Such segregation ensures that ligand and receptor engage only upon a loss of epithelial integrity. The ligands then reach their receptors and activate the subsequent signaling events that repair the injury and restore the epithelium. The basolateral release of VGF from infected epithelia suggests that vaccinia virus has developed a way of

activating basolateral erbB1 without a loss of epithelial integrity. These observations also suggest that investigating the similarities and differences between VGF and EGF might provide insight into the cellular mechanisms governing the polarity of their secretion.

**Additional considerations.** Our use of primary differentiated cultures offered an advantage over studies of cultured cell lines for studying virus-epithelium interactions. The use of polarized cells and an intact epithelium proved critical in determining the membrane involved in virus entry and release as well as the direction of VGF secretion. Growth of epithelia at the air-liquid interface also created an environment that resembles the *in vivo* airway surface. In addition, the use of human cells allowed us to preserve potential interactions that may be specific to the human host.

A limitation of this model is the lack of an acquired immune system that plays an important role during infection and could modulate the epithelial response to infection (45). In addition, viral shedding may be greater from the oropharynx than in the airways. The titers of infectious particles shed from the oropharynx are estimated to be 10- to 100-fold higher than titers originating from the airways (1, 53). Tonsillar epithelium is a stratified epithelium, as opposed to the pseudostratified columnar airway epithelium. Thus, it is possible that virus infection of that epithelium might show differences from those that we report here.

A previous study suggested that inhibiting the erbB1 receptor might be therapeutically beneficial for smallpox (55). An erbB1 inhibitor, CI-1033, administered to mice inoculated intranasally with vaccinia virus improved survival compared to untreated infected mice. This result is consistent with our findings that VGF and erbB1 play an important role in infection. However, our data raise the interesting possibility that VGF inhibition might lead to more airway epithelial destruction. Ultimately, the effect on survival will reflect a balance between the disruption of the virus life cycle and the response of host tissues.

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